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Noncanonical Wnt signaling in stromal cells regulates B-lymphogenesis through interleukin-7 expression

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ABSTRACT

The regulation of early B cell development and the interaction of hematopoietic precursors with stromal cells in the bone marrow (BM) are controlled by various secreted signaling molecules. Several recent studies showed Wnt signaling involved in B-lymphogenesis through stromal cells. However, the molecules modulated by Wnt signaling in stromal cells regulating B-lymphogenesis have not been identified yet. Interleukin (IL)-7 and CXC chemokine ligand (CXCL) 12 are known to be express in stromal cells, and both molecules are essential for B-lymphogenesis. In the present study, we examined the role of Wnt signaling in regulating IL-7 and CXCL12 expression and in affecting B-lymphogenesis. In mouse stromal ST2 cells, expression of IL-7 and CXCL12 mRNA was augmented by noncanonical Wnt5a. When mouse BM-derived cells were cultured on Wnt5a-overexpressing ST2 cells, an increased number of B220+/IgM- B-lymphoid precursor cells was observed. These results show that Wnt5a regulates IL-7 gene expression in stromal cells and suggest the possibility that noncanonical Wnt regulates B-lymphogenesis via IL-7 expression in stromal cells.

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1. Introduction

B cells are generated from hematopoietic stem cells (HSCs) in the bone marrow (BM) after birth, and they migrate into the blood to reach secondary lymphoid tissues, such as the spleen and lymph nodes in mammals. The early development of B cells in the BM is dependent on supportive microenvironment consisting of stromal cells known as niches that maintain blood cells and supply factors for their development [1,2]. Regulation of early B cell development and stromal interactions of hematopoietic precursors are thought to be controlled by various secreted signaling molecules, particularly, cytokines, chemokines, and growth factors [3].

Wnt secreted proteins are powerful regulators of embryonic development, cell differentiation, and proliferation [4,5], and they can activate two pathways: the β-catenin-dependent canonical and the β-catenin-independent noncanonical pathway. Noncanonical Wnt ligands activate the Wnt/Ca^{2+} pathway and Wnt/planar cell polarity (PCP) pathway [6]. It has been reported that receptor tyrosine kinase-like orphan receptor 2 (Ror2), a member of the Ror-family of receptor tyrosine-protein kinases, acts as a receptor or coreceptor for Wnt5a [7]. Ror2 by itself or in combination with Frizzled protein through its Frizzled-like cysteine-rich domain mediates diverse Wnt5a signaling by activating the Wnt–c-Jun N-terminal kinase PCP pathway [8].

Several studies have shown that Wnt signaling pathway is involved in the regulation of B-lymphogenesis in the hematopoietic microenvironment, the BM [9]. Canonical Wnt3a-stimulated stromal cells negatively regulated hematopoiesis, including early B-lymphogenesis. In contrast, noncanonical Wnt5a-producing stromal cells enhanced B-lymphopogenesis in culture [10,11]. However, the molecules modulated by the Wnt signaling in stromal cells and involved in early B-lymphogenesis have not been characterized yet.

Interleukin (IL)-7 and CXC chemokine ligand (CXCL) 12 (stromal cell-derived factor-1/pre-B-cell-growth-stimulating factor), which are supplied by stromal cells, are well known to play crucial and essential roles in B-lymphogenesis [12,13]. Both molecules were identified and characterized by *in vitro* coculture system using several stromal cell lines, such as ST2 and PA6 cells [14]. The *in vivo* studies using mutant mice with targeted gene disruption have revealed that CXCL12 and IL-7 expression on stromal cells are essential for B-lymphogenesis [15]. Also, IL-7 and IL-7 receptor α-chain (IL-7Rα)–deficient mice revealed impaired Bcell development due to early B-cell progenitors [16,17]. To date, little is known on the regulation of IL-7 production, especially in stromal cells that are considered the main source of this cytokine.

Several growth factors and cytokines are known to modulate B-lymphogenesis via the regulation of IL-7 and CXCL12 expression on stromal cells. Tang et al. [18] showed that transforming growth

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factor (TGF)-β downregulates IL-7 secretion in stromal cells and inhibits proliferation of Bcell precursors [18]. Also, TGF-β1 downregulates CXCL12 expression in the stromal cell line MS-5 [19]. Mice deficient of G protein α subunit (GSα), which is a major downstream activator of the parathyroid hormone- related peptide receptor signaling in osterix-expressing stromal cells, specifically showed a failure of B-lymphopogenesis through the reduction of IL-7 production in stromal cells [20]. Our previous study demonstrated that canonical Wnt3a regulates CXCL12 expression in ST2 cells [21]. However, the role of Wnt signaling in the regulation of IL-7 expression in stromal cells and in the development of B cells remains unclear.

In this study, we examined the effects of Wnt signaling on the regulation of IL-7 expression in ST2 cells, and then on B-lymphogenesis using an *in vitro* coculture system. Wnt5a enhanced IL-7 expression in ST2 cells and increased the number of Bcell progenitors. These findings demonstrate that noncanonical Wnt signaling in stromal cells regulates B-lymphogenesis partially through IL-7 expression.

2. Materials and methods

2.1. Murine BM cells

Murine adherent cell-depleted BM cells were isolated from seven-week-old C57BL/6J mice from Nippon Clea (Tokyo, Japan). The experiments were performed in accordance with the guidelines on the care and use of laboratory animals and have been approved by Hokkaido University.

2.2. Cell cultures

ST2 cells were obtained as described previously [21]. Wnt3a-ST2 and Wnt5a-ST2 cells were established as described previously [21]. Cells were grown to semiconfluence in alpha minimum essential medium (α-MEM) (Sigma-Aldrich, St. Louis, MO, USA) containing 100 μg/mL kanamycin (Meiji, Tokyo, Japan) and supplemented with 10% fetal bovine serum (FBS; PAA Laboratories; Pasching, Austria) at 37 °C (Corning, Corning, NY, USA) in a humidified atmosphere of 5% CO_2. The medium was removed, and 1×10^6 adherent cell-depleted BM cells were cultured with or without ST2, Wnte3a-ST2, or Wnt5a-ST2 cell layer in RPMI1640 medium (Sigma-Aldrich) supplemented with 5% FBS and 50 μM 2-mercaptoethanol at 37 °C for 4, 5, or 7 days. Floating cells were analyzed by flow cytometry.

2.3. Reagents

Mouse recombinant Wnt5a was obtained from R&D Systems Inc. (Minneapolis, MN, USA).

2.4. Flow cytometry

Flow cytometry analysis was carried out using the following antibodies: PE-anti-B220, PE-anti-CD3ε and PE-anti-CD11b from BD Bioscience (BD Bioscience, San Jose, CA). Stained cells were analyzed for surface expression using a flow cytometer (FACSCalibur; BD Biosciences) and analyzed with CellQuest software (BD Biosciences) as described previously [22].

2.5. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from the cells using Isogen (Nippongene, Toyama, Japan), and RT-PCR was performed as previously described [23]. All the primers were synthesized by Hokkaido System Science (Sapporo, Japan). The primer sequences were described previously [24].

2.6. Quantification of gene expression by quantitative RT-PCR (qRT-PCR)

Total RNA was reverse transcribed using first-strand cDNA synthesis with random primers (Promega, Madison, WI, USA). The PCR was performed using SYBER Green (Invitrogen Life Technologies Carlsbad, CA, USA) and ABI StepOne Plus real-time PCR system (Applied Biosystems, Foster City, CA, USA). Primer sequences for each gene were the following: CXCL12, 5′-CAACACTCCAAACTGTGCCCTTCA-3′ (forward), 5′-TCCTTTGGGCTGTTGTGCTTACT-3′ (reverse); IL-7, 5′-TGGGAGTGATTATGGGTGGT-3′ (forward), 5′-GCGAGCAGCACGATTTAGAAAAGC-3′ (reverse); thymic stromal lymphopoietin (TSLP), 5′-AGGCTACCCTGAAACTGAG-3′ (forward), 5′-GGAGATTGCATGAAGGAATACC-3′ (reverse); β-actin; 5′-CTTCTTTGCAGCTCCTTCGTTG-3′ (forward), 5′-CGACCAGCGCAGCGATATC-3′ (reverse). The relative level of gene expression was quantified using the comparative Ct method with β-actin as the endogenous control.

2.7. Cell proliferation assay

To quantify cell proliferation, the tetrazolium-based colorimetric CCK-8 assay (Dojindo Laboratories, Kumamoto, Japan) was used. A 20 μL aliquot of the substrate WST-8 (2-[2-methoxy-4-nitrophenyl]-3-[4-nitrophenyl]-5-[2,4-disulfophenyl]-2H-tetrazolium, monosodium salt) was added to each well. After incubation for 2 h at 37 °C, the optical density was measured at a wavelength of 450 nm using a microplate reader (iMark, Bio-Rad Laboratories, Richmond, CA, USA).

2.8. Statistical analysis

All experiments were repeated three to four times and representative results are shown. The data are reported as the mean ± standard deviation (SD) and were analyzed by Student's *t*-test, where p values < 0.05 were considered significant.

3. Results

3.1. Wnt signaling regulates IL-7 and CXCL12 expression in ST2 cells

To evaluate the role of Wnt signaling on IL-7 and CXCL12 regulation, which is essential for B-lymphogenesis in stromal cells, we examined their mRNA expression using qRT-PCR. We had previously established stromal ST2 cells expressing either Wnt3a (Wnt3a-ST2 cells), which stimulates canonical Wnt signaling, or Wnt5a (Wnt5a-ST2 cells), which stimulates noncanonical Wnt signaling [21]. IL-7 mRNA expression level was upregulated in both Wnt3a-ST2 and Wnt5a-ST2 cells, particularly in Wnt5a-ST2 cells, compared with vehicle-transfected ST2 cells (Fig. 1A). CXCL12 mRNA level was increased in Wnt5a-ST2 cells, whereas it was reduced in Wnt3a-ST2 cells. However, mRNA expression of the IL-7-related cytokine TSLP, the receptor formed by a heterodimer of IL-7Rα and another receptor subunit, was altered neither by Wnt3a nor by Wnt5a overexpression in ST2 cells (Fig. 1A). Treatment with the recombinant Wnt5a protein (200 ng/mL) also significantly increased IL-7 mRNA expression in ST2 cells (Fig. 1B). To confirm the increment of IL-7 mRNA expression by Wnt5a in another cell line, we examined Wnt5a-C2C12 cells, previously established [23]. In Wnt5a-C2C12 cells, IL-7 mRNA expression level was 1.75 times higher than that in C2C12 cells (data not shown; relative average value, n=6). These results indicate that

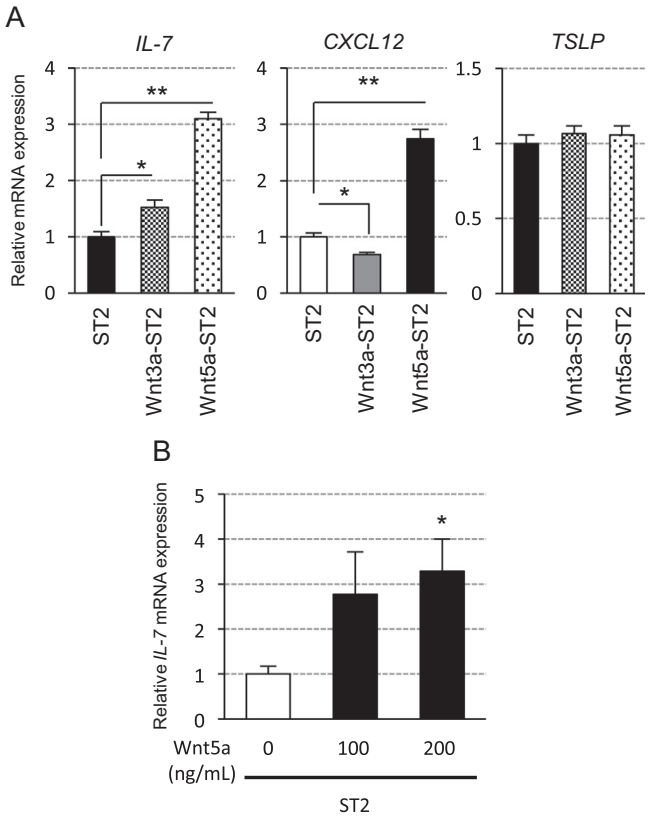

Fig. 1. Noncanonical Wnt signaling modulates interleukin (IL)-7 gene expression in ST2 cells. (A) ST2, Wnt3a-ST2, or Wnt5a-ST2 cells were cultured to semiconfluence, and total RNA was extracted. IL-7 (left), CXCL12 (center), and thymic stromal lymphopoietin (TSLP) (right) mRNA expression levels were determined by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). (B) ST2 cells were plated at a density of 2×10^5 cells/mL in 6-well culture plates and cultured for 24 h. Indicated doses of Wnt5a were added, and the cells were cultured for further 48 h. Total RNA was extracted from the cells, and IL-7 mRNA expression level was determined by qRT-PCR. Data are presented as means ± S.D. *, $p < 0.05$, **, $p < 0.01$.

noncanonical Wnt5a signaling augments IL-7 mRNA expression; then the activation of noncanonical Wnt signaling in stromal cells may affect B-lymphogenesis through the regulation of IL-7 expression.

3.2. IL-7 and CXCL12 expression are important for the maintenance of B cells in a coculture system

To determine the ability of ST2 cells to maintain B cells in a coculture system, mouse BM cells were cultured on layers of different adherent cells, such as MC3T3-E1, MLO-Y4, and ST2 cells under inducible B-lymphogenesis condition. Among these adherent cells only ST2 cells could support B cells, and after 4 days we could detect B220-positive B cells among floating cells (Fig. 2A). To evaluate whether ST2 cells were able to maintain B cells during long-term coculture systems, number of floating cells and of several types of hematopoietic cells, such as B220-positive B cells, CD3ε-positive T cells and CD11b-positive myeloid cells were examined. In this culture system, ST2 cells were able to maintain B220-positive B cells for 7 days, but not CD3ε-positive T cells and CD11b-positive myeloid cells (Fig. 2B). ST2 cells highly expressed IL-7 and CXCL12 mRNA compared with other adherent cells (Fig. 2C), indicating that IL-7 and CXCL12 expression in ST2 cells may be important for long-term B cell coculture systems.

3.3. Regulation of the ability of ST2 cells in supporting B cells by noncanonical Wnt signaling

To examine whether the change in IL-7 and CXCL12 expression in ST2 cells by noncanonical Wnt signaling affected the ability to maintain B cells, we cultured mouse BM cells on the layer of ST2, Wnt3a-ST2, or Wnt5a-ST2 cells. After 5 or 7 days of culture, we analyzed the number of B220-positive IgM-negative B-cell progenitors and of B220-positive B cells in floating hematopoietic cells by flow cytometry. The number of B220-positive IgM-negative B-cell progenitors cultured for 7 days on Wnt5a-ST2 cells significantly increased and also the number of progenitors cultured on Wnt3a-ST2 cells slightly increased but not significantly. The percentage of B220-positive IgM-negative B-cell progenitors cultured on both Wnt3a-and Wnt5a-ST2 cells were significantly increased (B220+/IgM− B cell progenitors on ST-2 cells; 42.21 ± 1.9%, Wnt3a-ST2 cells; 51.60 ± 1.7%, Wnt5a-ST2 cells; 55.12 ± 2.4%). This result indicated that also canonical Wnt signaling in stromal cells may regulate B cell differentiation with or without modulation of IL-7. On the other hand, the number of B220-positive B cells cultured for 5 or 7 days on Wnt3a-ST2 cells or Wnt5a-ST2 cells did not change (Fig. 3A). To elucidate whether the activation of Wnt signaling modulated cell proliferation and affected B-lymphogenesis, we performed a cell proliferation assay. The number of vital Wnt3a-ST2 and Wnt5a-ST2 cells cultured for 3 days was not altered (Fig. 3B). These results suggest that Bcell progenitors were maintained by noncanonical Wnt signaling in stromal cells probably through the upregulation of IL-7 expression.

4. Discussion

In the present study, noncanonical Wnt5a enhances IL-7 mRNA expression in stromal cells. IL-7 is produced by stromal cells residing in the primary and secondary lymphoid organs, and its receptor (IL7R), consisting of an IL7Rα chain and a common γ chain, is expressed in lymphoid cells and is critical for lymphogenesis. IL-7– and IL-7Rα–deficient mice show a block in early B-cell development at the uncommitted B cell stage [16,17]. In early B-cell development, it is generally thought that the IL7R and the pre-B cell antigen receptor synergistically activate both the MAPK/Erk and the PI3K signaling pathway, resulting in enhanced cell proliferation and survival [3]. Our results suggest that the noncanonical Wnt5a functionally enhances IL-7 production in ST2 cells and that IL-7 then regulates early Bcell expansion in a coculture system. Previous studies showed that IL-7 also plays a critical role in T-cell development in primary and secondary lymphoid organs [25]. Regulation of IL-7 expression by noncanonical Wnt signaling may contribute to not only B-cell but also T-cell development.

Hematopoiesis is regulated by autocrine and paracrine mechanisms. Many Wnt family proteins are expressed in hematopoietic tissues, and a series of reports suggest that they have important roles in not only the maintenance of HSCs in the niche but also in the development of hematopoietic cells, including B cells [26]. Wnt signaling regulates B-lymphogenesis via both cell intrinsic and cell extrinsic mechanisms in primary lymphoid organs. A previous study reported that Wnt5a-deficient mice showed increased B cell proliferation [27]. However, this B-lymphogenetic abnormality was intrinsic to the hematopoietic cells, since abnormality of Wnt5a−/− hematopoietic cells could not be rescued by transplantation of recipient mice whose BM stromal cells expressed Wnt5a [27]. Our results using coculture systems have the possibility that Wnt3a- or Wnt5a-transfected ST2 cells secrete the Wnt proteins, thus regulating IL-7 expression and affecting early B-lymphogenesis. Consistent with our results, Wnt5a conditioned medium or Wnt5a overexpressing stromal cells also

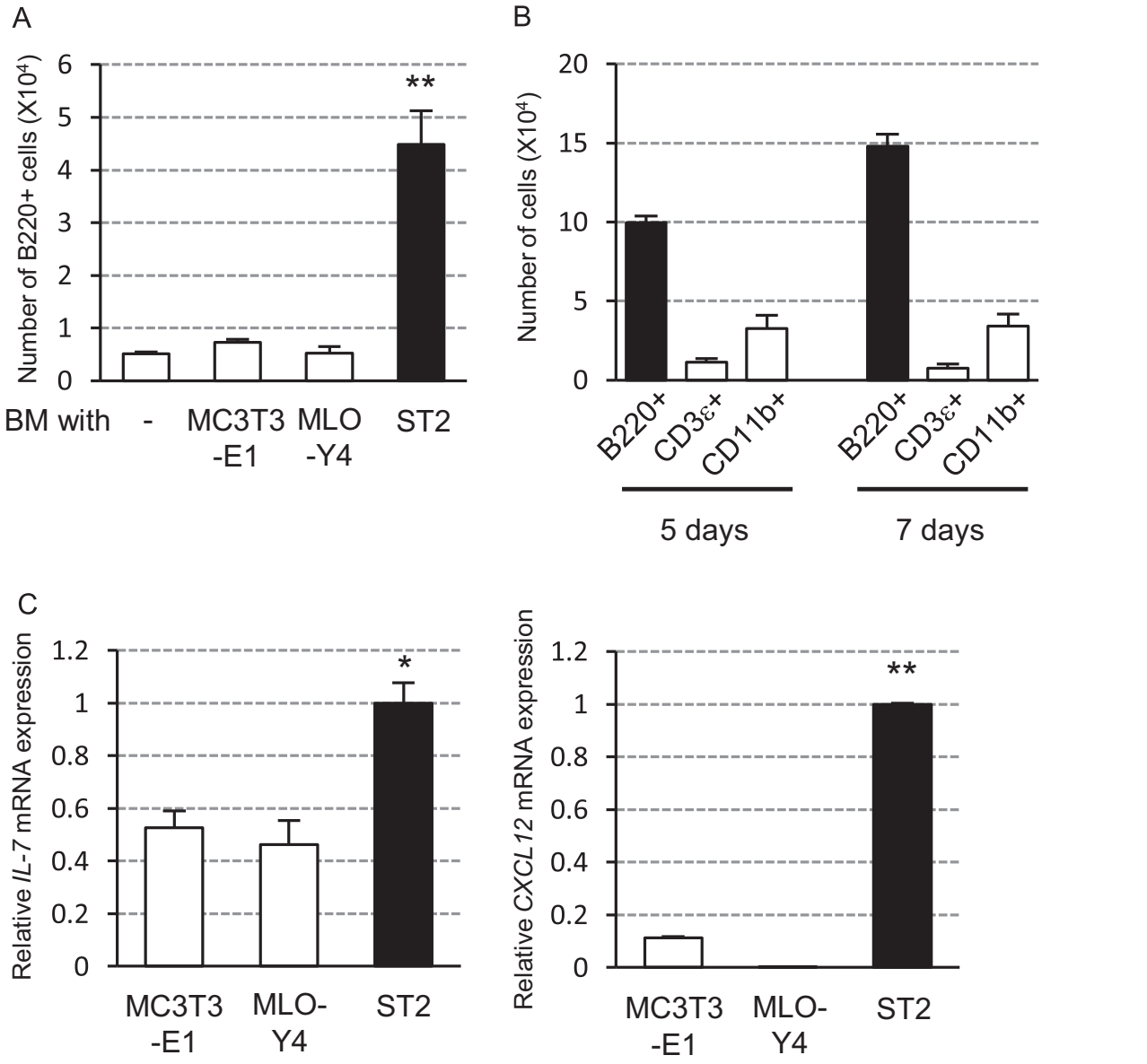

Fig. 2. ST2 cells maintained B cells in a coculture system. (A and B) Murine bone marrow (BM) cells were cultured with indicated cell lines for 4 days. Floating cells were analyzed for B220, CD3ε, and CD11b expression by FACS. (C) Indicated cells were cultured to semiconfluence, and then total RNA was extracted. IL-7 (left) and CXCL12 (right) mRNA expression levels were determined by qRT-PCR. Data are presented as means ± S.D. *, $p < 0.05$.

enhanced early B cell development in coculture systems [10,11]. Taken together, these data indicate that B-lymphogenesis might be regulated by noncanonical Wnt ligand through cell extrinsic mechanisms, such as regulation of IL-7 and CXCL12 expression in stromal cells.

To elucidate the regulation of hematopoiesis through the BM niche, niche cell compartment and identification of factors derived from niche cells are important because they regulate BM hematopoiesis. Two distinct niches have been identified in the BM: the vascular niche and the osteoblastic niche. The vascular niche may play important roles in regulating HSC mobilization and in retaining the cells in a quiescent state. Visnjic et al. showed that the osteoblast ablation resulted in the alteration of hematopoiesis [28]. Also, the deletion of CXCL12 from osterix positive stromal cells or col2.3 positive osteoblasts resulted in the depletion of B lymphocytes [29,30]. These studies suggest that the osteoblastic niche may be composed by CXCL12-expressing stromal cells, which include osteoblast precursors and support B lymphocyte commitment and differentiation from HSC [29,30]. In this study, we used ST2 cells as stromal cells, and we showed that ST2 cells could maintain hematopoietic cells in coculture system. ST2 cells cultured with osteoblastic inducers, including ascorbic acid and dexamethasone, exhibited characteristics typical of osteoblasts, such as formation of mineralized nodules [31], thus indicating that ST2 cells are similar to pre-osteoblasts [31]. Taken together, these data indicate that ST2 cells are similar to osteoblastic niche cells *in vivo*, thus suggesting that our *in vitro* culture studies on B-lymphogenesis regulation through the change of IL-7 and CXCL12 expression in ST2 cells by Wnt signaling may elucidate the regulatory system in the B-lymphoid niche *in vivo*.

During hematopoiesis, several studies showed the influence of Wnt signaling on niche cells in the BM. Osteoblastic niche cells predominantly express noncanonical Wnt ligands such as Wnt5a [32]. Also, Wnt signaling plays an important role in bone metabolism by controlling differentiation of osteoblasts and osteoclasts. One key implication of our data is that B-cell development is directly linked to the noncanonical Wnt signaling in the stromal cells, indicating that noncanonical Wnt signaling in the BM might

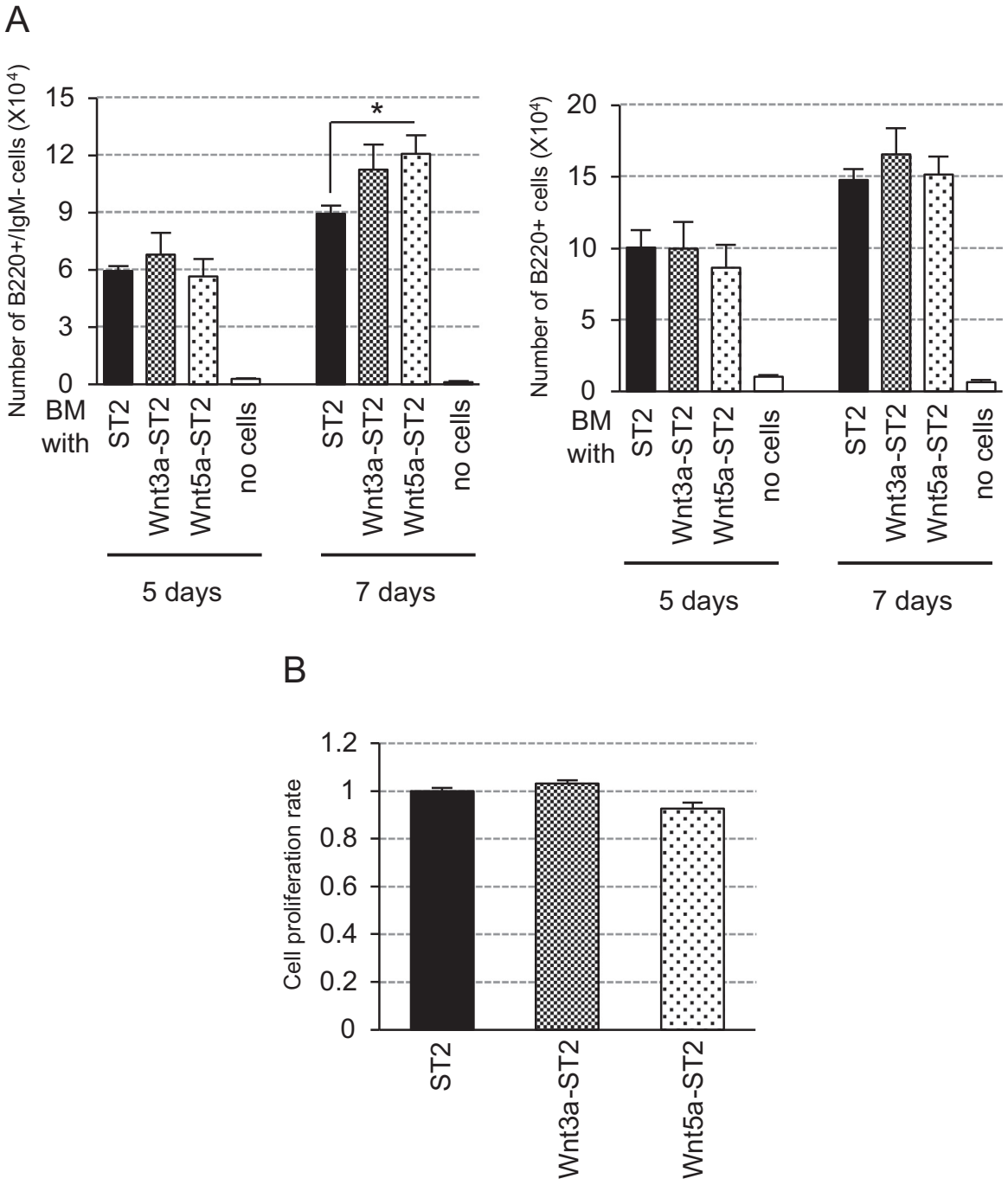

Fig. 3. Noncanonical Wnt signaling in ST2 cells regulates B-lymphogenesis. (A) Murine BM cells were cultured with the indicated cell lines for the indicated times. Floating cells were analyzed for B220 or IgM expression by FACS. (B) ST2, Wnt3a-ST2, or Wnt5a-ST2 cells were plated at a density of 0.5×10^5 cells/well in 24-well plates and cultured for further 72 h. The proliferating cells were quantified as described in the Section 2. The relative numbers of ST2 cells are adjusted to 1. Data are presented as means $\pm$ S.D. *, $p < 0.05$.

contribute to B-lymphogenesis partly through IL-7 expression. Hardy et al. reported that B220 positive and IgM negative immature B cells divided into three populations and they showed phenotypic distinctions. Interestingly, cell proliferation of all of immature B cell populations dependent on the presence of stromal cells and two of populations response to IL-7. In another population, less responsive to IL-7, of immature B cells, their proliferation was absolutely depend on stromal cell contact and some cell contact-mediating signals seem to be essential for survival [33]. Thus the role of IL-7 induced by noncanonical Wnt signaling in stromal cells for B-lymphogenesis may be partial and other factors including CXCL12 are also involved for B-lymphogenesis. Overall, our results suggest that the further characterization of the effects of Wnt signaling in B-lymphogenesis through cell extrinsic mechanism will elucidate the local mechanisms regulating stem cell lineage commitment and differentiation.

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Appendix A. Transparency document

Transparency document associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2016.03.017.

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